IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Serial No. : 09/925,673

Applicants : Masakatsu KANEKO et al.

Filed : August 9, 2001

For : NOVEL NUCLEOSIDE AND OLIGONUCLEOTIDE ANALOGUES

Art Unit : 1623

Examiner : Ganapathy Krishnan

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DECLARATION UNDER 37 CFR 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

MAIL STOP AMENDMENT

S I R :

I, Makoto Koizumi, declare as follows:

A. My Education and Experience

1. I graduated from Hokkaido University, Sapporo, Japan, in the year 1986, and I received a doctorate degree from Hokkaido University on the studies of catalytic ribozymes with sequence-specific RNA cleaving activity in 1991.

2. I have worked for Sankyo Company, Limited, Tokyo, Japan, since 1991. My research activities at Sankyo Company, Limited have included the following: synthesis of nucleoside

analogs with antibiotic activity and synthesis of modified oligonucleotides with antivirus, anticancer and anti-diabetes activity. My research activities were not limited to those in the company; I studied as a visiting researcher at Yale University, New Haven, CT, for two years from November 1997. I presently hold the position of the Chief Researcher of Core Technology Research Laboratories of Sankyo Company, Limited. I have held this position since the year 2003.

3. I am a member of the Pharmaceutical Society of Japan. I am on the committee of Antisense DNA/RNA Society, Japan.

4. I have contributed many scientific papers. For example, I am a co-author of "Biologically active oligodeoxyribonucleotides. 5. 5'-End-substituted d(TGGGAG) possesses anti-human immunodeficiency virus type 1 activity by forming a G-quadruplex structure." *J. Med. Chem.* 1998, 41, 3655-3663; "Allosteric selection of ribozymes that respond to the second messengers cGMP and cAMP." *Nat. Struct. Biol.* 1999, 6, 1062-1071. Also, I am a corresponding author of "Synthesis and properties of 2'-O,4'-C-ethylene-bridged nucleic acids (ENA) as effective antisense oligonucleotides." *Bioorg. Med. Chem.* 2003, 11, 2211-2226; "Triplex formation with 2'-O,4'-C-ethylene-bridged nucleic acids (ENA) having C3'-endo conformation at physiological pH." *Nucleic Acids Res.* 2003, 31, 3267-3273; "Direct comparison of in vivo antisense activity of ENA oligonucleotides targeting PTP1B mRNA with that of 2'-O-(2-methoxy)ethyl-modified oligonucleotides." *Oligonucleotides* 2006, 16, 253-262.

5. I am named as an inventor in many patents issued in Japan, the United States and other countries. I am listed as an inventor in the following US patents: "Looped hairpin ribozyme." U.S. Patent 5,631,115 (issued May 20, 1997); "Modified oligodeoxyribonucleoditides." U.S. Patent 5,674,856 (issued October 7, 1997); "Composition and method for the treatment or

prophylaxis of viral infections using modified oligodeoxyribonucleotides." U.S. Patent 5,807,837 (issued September 15, 1998).

6. I was the recipient of the following academic and professional awards: "JB award, the Japanese Biochemical Society, 1996"; "Bioorganic Medicinal Chemistry Most Cited Paper 2003-2006 Award, 2006".

7. I am an expert in the way and manner of selecting and synthesizing compounds suitable for development as pharmaceuticals based on the chemistry and biology of nucleosides, nucleotides and oligonucleotides.

B. The experiments set forth in the attachments hereto entitled Certificate of Experimental Results (2), Certificate of Experimental Results (3), Certificate of Experimental Results (5) and Certificate of Experimental Results (6) were carried out under my supervision. These attachments were submitted in the European Patent Office in the corresponding European patent application and relate to issues raised in said European patent application.

The results set forth in the attachments hereto show unexpected results for ENA compounds of the present claims compared to LNA (BNA) compounds such as in Wengel et al.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title

18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: Nov/6/2006 _____ _____

Makoto KOIZUMI

Certificate of Experimental Results (2)

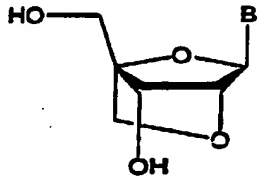
(Abstract)

Compounds of this invention were evalnated for nuclease P_1 (endonuclease) resistance. From the results, it was clear that this invention has an inventive step in the light of the prior arts cited in the Official Report.

(Methods and Results)

(a) Synthesis of a reference compound

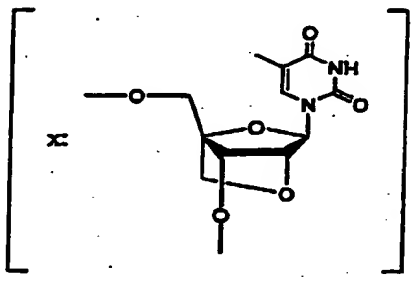
The four citations cited by the examiner contain a 2'-*O*,4'-*C*-methylene nucleoside having the following structure (I).



(I)

Oligonucleotide D, which contains the 2'-*O*,4'-*C*-methylene nucleoside of (I), was synthesized according to the method described in WO98/39352 and its nuclease resistance was compared with oligonucleotides containing the invented nucleosides. The purified oligonucleotide D was analyzed by Reverse phase HPLC (column: Wakosil DNA 4.6 x 150 mm, solution A: 5% acetonitrile, 0.1M triethylammonium acetate (TEAA, pH 7.0), solution B: 25% acetonitrile 0.1 M TEAA (pH 7.0), B% 10 – 50% (28 min), flow rate: 1 ml/min, column temp. 60°C, 260 nm). Retention time: 22.82 min. The structure of Oligonucleotide D was determined by negative-ion ESI mass spectroscopy. calcd: 877.63, found: 877.41.

Oligonucleotide D: 5'-txt-3'



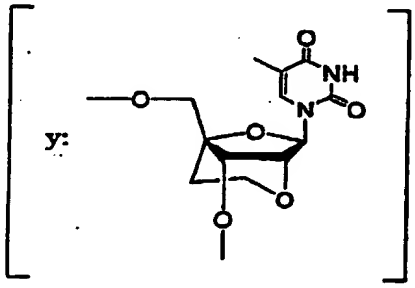
Oligonucleotide E, which consisted of thymidine units, was also synthesized according to the standard method. The purified oligonucleotide E was analyzed by Reverse phase HPLC (column: Wakosil DNA 4.6 x 150 mm, solution A: 5% acetonitrile, 0.1M triethylammonium acetate (TEAA, pH 7.0), solution B: 25% acetonitrile 0.1 M TEAA (pH 7.0), B% 10 – 50% (28 min), flow rate: 1 ml/min, column temp. 60°C, 260 nm). Retention times: 24.70 min. The structure of Oligonucleotide E was determined by negative-ion ESI mass spectroscopy. calcd: 849.62, found: 849.44.

Oligonucleotide E: 5'-ttt-3'

(b) Synthesis of a compound of this invention

Oligonucleotide F, which contains the invented nucleosides, was synthesized according to the method described in the specification of this application. The purified oligonucleotide F was analyzed by Reverse phase HPLC (column: Wakosil DNA 4.6 x 150 mm, solution A: 5% acetonitrile, 0.1M triethylammonium acetate (TEAA, pH 7.0), solution B: 25% acetonitrile 0.1 M TEAA (pH 7.0), B% 10 – 50% (28 min), flow rate: 1 ml/min, column temp. 60°C, 260 nm). Retention times: 26.53 min. The structure of Oligonucleotide F was determined by negative-ion ESI mass spectroscopy. calcd: 891.66, found: 891.45.

Oligonucleotide F: 5'-tyt-3'



(c) Assay method of nuclease resistance of oligonucleotides and results

We tested the resistance of oligonucleotide D, oligonucleotide E and oligonucleotide F against nuclease P_1. A 15-μg of each oligonucleotide was added to 2250 μl of a solution containing 50 mM sodium acetate (pH 5.2) and 0.1 mM $ZnCl_2$ (final concentration of the oligonucleotides: 6.4 μg/ml), 75 μl of 40 μg/mL nuclease P_1 (This solution was prepared by dilution of 2 mg/mL nuclease P_1 (Sigma) with sterilized water and was used immediately after this preparation. Final concentration of nuclease P_1: 1.3 μg/ml) was added and then incubated at 37°C. At the time points of 0, 30 and 60 min, 360-μl aliquots were taken and immediately were heated at 90°C for 4 min. 300-μl of reaction

mixtures were taken as samples for HPLC analyses. Reverse phase HPLC analyses were carried out with a gradient of acetonitrile and a constant of 0.1 M triethylammonium acetate (pH 7.0) (column: Wakopak WS-DNA 4.6 x 150 mm, flow rate: 1 ml/min, 254 nm). The nuclease-resistance activity was defined as the percent ratio of remaining oligonucleotides compared with the initial levels. The results are shown in Table 1.

Table 1. Percentage of remaining oligonucleotides.

Sample	0 min	30 min	60 min
Oligonucleotide D	100	52	34
Oligonucleotide E	100	not detected	not detected
Oligonucleotide F	100	84	76

(Discussion)

Oligonucleotide D of the prior art had a higher nuclease-resistance activity than oligonucleotides E, which is a natural oligothymidylate. Although 34% of oligonucleotide D was detected after 60 min of incubation, 76% of Oligonucleotide F still remained. The initial rate constants of hydrolysis of Oligonucleotide D and Oligonucleotide F were calculated as 1.8×10^{-2} min^{-1} and 4.6×10^{-3} min^{-1}, respectively. It is found that Oligonucleotide F was approximately 4 times more stable than Oligonucleotide D. It turned out that oligonucleotide F of this invention had a much higher nuclease-resistance activity than oligonucleotide D of the prior art. The remarkably high nuclease-resistance activity of a compound of this invention is not obvious for the person skilled in the art. Since the role of the ring structure of the invented nucleosides is not only structural, they are thought to have an inventive step in the light of the prior arts.

Certificate of Experimental Results (3)

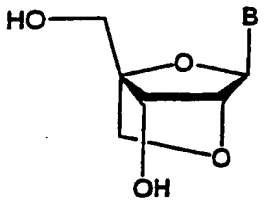
(Abstract)

Compounds of this invention were evaluated for stability in rat plasma. From the results, it was clear that this invention has an inventive step in the light of the prior arts cited in the Official Report.

(Methods and Results)

(a) Synthesis of a reference compound

The four citations cited by the examiner contain a 2'-*O*,4'-*C*-methylene nucleoside having the following structure (I).

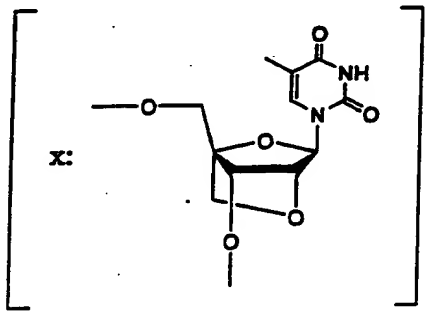


(I)

Oligonucleotide G and Oligonucleotide H, which contain the 2'-*O*,4'-*C*-methylene nucleoside of (I), were synthesized according to the method described in WO98/39352 and their stability was compared with oligonucleotides containing the invented nucleosides.

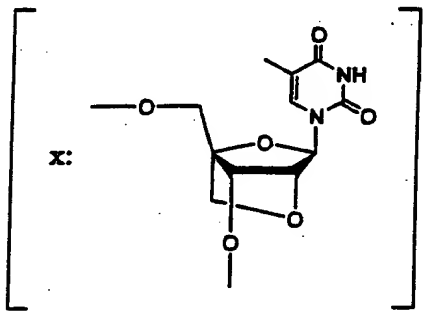
The purified oligonucleotide G was analyzed by anion exchange HPLC (column: Tosho DEAE-5PW 7.5 x 75 mm, solution A: 20% acetonitrile, solution B: 20% acetonitrile, 67 mM phosphate buffer (pH 6.8), 1.5 M NaCl, B%: 18% isocratic, flow rate: 2 ml/min, 260 nm). Retention time: 10.15 min. The structure of Oligonucleotide D was determined by negative-ion ESI mass spectroscopy. Calcd 2880.12, found 2878.30.

Oligonucleotide G: 5'-xxts tststs xxx-3'



The purified oligonucleotide H was analyzed by anion exchange HPLC (column: Tosho DEAE-5PW 7.5 x 75 mm, solution A: 20% acetonitrile, solution B: 20% acetonitrile, 67 mM phosphate buffer (pH 6.8), 1.5 M NaCl, B%: 18% isocratic, flow rate: 2 ml/min, 260 nm). Retention time: 10.23 min. The structure of Oligonucleotide H was determined by negative-ion ESI mass spectroscopy. Calcd 2815.85, found 2814.40.

Oligonucleotide H: 5'-xtx txt xtx-3'



Oligonucleotide I, which consisted of thymidine units, was synthesized according to the standard method. The purified oligonucleotide I was analyzed by Reverse phase HPLC (column: Wakosil DNA 4.6 x 150 mm, solution A: 5% acetonitrile, 0.1M triethylammonium acetate (TEAA, pH 7.0), solution B: 25% acetonitrile 0.1 M TEAA (pH 7.0), B% 30 – 70% (20 min), flow rate: 1 ml/min, column temp. 60°C, 260 nm). Retention time: 6.39 min. The structure of Oligonucleotide I was determined by negative-ion ESI mass spectroscopy. Calcd 2675.80, found 2674.41.

Oligonucleotide I: 5'-ttt ttt ttt-3'

Oligonucleotide J, which consisted of thymidine units and phosphorothioate linkages, was also synthesized according to the standard method. The purified oligonucleotide J was analyzed by Reverse phase HPLC Reverse phase HPLC (column: Wakosil DNA 4.6 x 150 mm, solution A: 5% acetonitrile, 0.1M triethylammonium acetate (TEAA, pH 7.0), solution B: 25% acetonitrile 0.1 M TEAA (pH 7.0), B% 30 – 70% (20 min), flow rate: 1 ml/min, column temp. 60°C, 260 nm) Retention time: 18.02 min. The structure of Oligonucleotide J was determined by negative-ion ESI mass spectroscopy. Calcd 2804.33, found 2802.25.

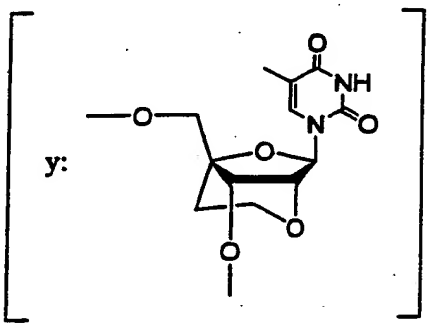
Oligonucleotide J: 5'-tststs tststs tststs-3'

(b) Synthesis of a compound of this invention

Oligonucleotide K and Oligonucleotide L, which contain the invented nucleosides, were synthesized according to the method described in the specification of this application.

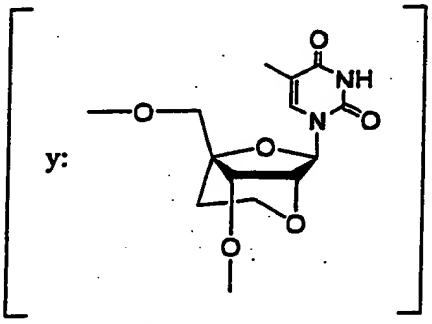
The purified oligonucleotide K was analyzed by Reverse phase HPLC (column: Wakosil DNA 4.6 x 150 mm, solution A: 5% acetonitrile, 0.1M triethylammonium acetate (TEAA, pH 7.0), solution B: 25% acetonitrile 0.1 M TEAA (pH 7.0), B% 30 – 70% (20 min), flow rate: 1 ml/min, column temp. 60°C, 260 nm). Retention times: 14.57 and 15.65 min. The structure of Oligonucleotide K was determined by negative-ion ESI mass spectroscopy. Calcd 2950.25, found 2948.38

Oligonucleotide K: 5'-yyts tststs yyy-3'



The purified oligonucleotide L was analyzed by Reverse phase HPLC (column: Wakosil DNA 4.6 x 150 mm, solution A: 5% acetonitrile, 0.1M triethylammonium acetate (TEAA, pH 7.0), solution B: 25% acetonitrile 0.1 M TEAA (pH 7.0), B% 30 – 70% (20 min), flow rate: 1 ml/min, column temp. 60°C, 260 nm). Retention times: 11.56 min. The structure of Oligonucleotide L was determined by negative-ion ESI mass spectroscopy. calcd 2885.99, found 2885.80.

Oligonucleotide L: 5'-yty tyt yty-3'



(c) Methods and Results Stability assay in rat plasma

Rat whole blood was obtained from adult male Sprague-Dawley rats (Charles River Japan). Plasma was separated by centrifugation at 3000 rpm for 10 min. A 20-μl aliquot of each

oligonucleotide (1 mg / ml) was added to 980 μl of the rat plasma (finally 20 μg/ml) and incubated at 37°C. At the time points of 0, 0.25, 0.5, 1 and 4 hr, 100-μl aliquots were taken and immediately were added with 300 μl methanol containing internal standard compound (5'-XCCCCCCCC-3': X = 2'-*O*-*tert*-butyldimethylsilyl uridine, C = 2'-deoxycytidine, 5 μg/ml) and centrifuged at 10,000 rpm at 4°C for 2 min to remove the proteins. The supernatants were taken as samples for HPLC analyses. Reverse phase HPLC analyses were carried out with a gradient of acetonitrile and a constant of 0.1 M triethylammonium acetate (pH 7.0) (column: Wakopak WS-DNA 4.6 x 150 mm, flow rate: 1 ml/min, 260 nm). The average residual amount of oligonucleotides and corresponding standard deviation were obtained from three independent experiments.

We compared the stability of Oligonucleotide K and Oligonucleotide L with those of Oligonucleotide G, Oligonucleotide H, Oligonucleotide I and Oligonucleotide J in rat plasma. Each oligonucleotide was incubated with rat plasma (final conc. 98%) at 37°C and the mixture was analyzed by reverse-phase HPLC. Oligonucleotide I was completely degraded in 15 min. Oligonucleotide K and Oligonucleotide L were more stable than corresponding Oligonucleotide G, and Oligonucleotide H, respectively as shown in Figure 1. Among the oligonucleotides we tested, Oligonucleotide K showed excellent stability, remaining almost intact, while Oligonucleotide J, of which half were degraded in 4 hr in rat plasma, did not.

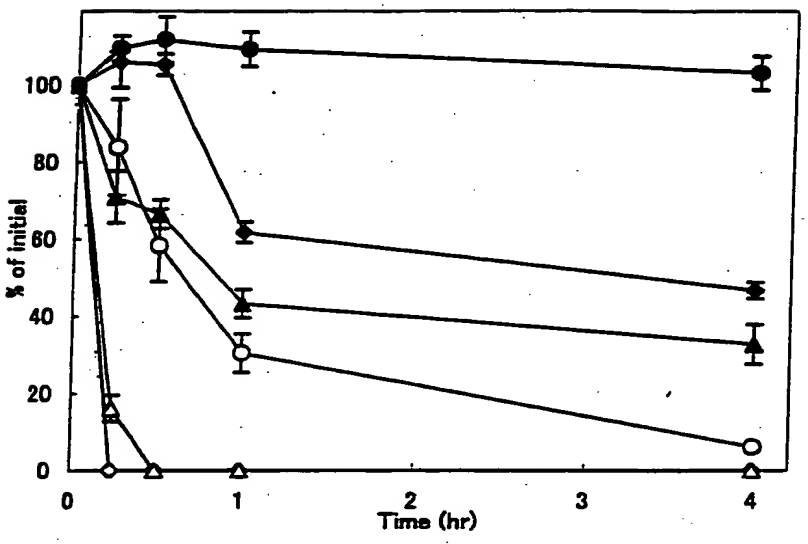


Figure 1. Stability of oligonucleotides in rat plasma.

closed triangles: Oligonucleotide G (5'- xxts tststs xxx-3')
open triangles: Oligonucleotide H (5'-xtx txt xtx-3')
open diamonds: Oligonucleotide I (5'-ttt ttt ttt-3')
closed diamonds: Oligonucleotide J (5'-tststs tststs tstst-3')
closed circles: Oligonucleotide K (5'-yyts tststs yyy-3')
open circles: Oligonucleotide L (5'-yty tyt yty-3')

Certificate of Experimental Results (5)

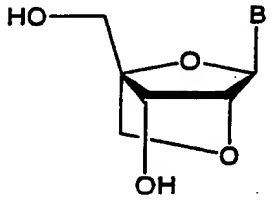
(Abstract)

Compounds of this invention were evaluated for exonuclease III resistance. From the results, it was clear that this invention has an inventive step in the light of the prior arts cited in the Official Report.

(Methods and Results)

(a) Synthesis of a reference compound

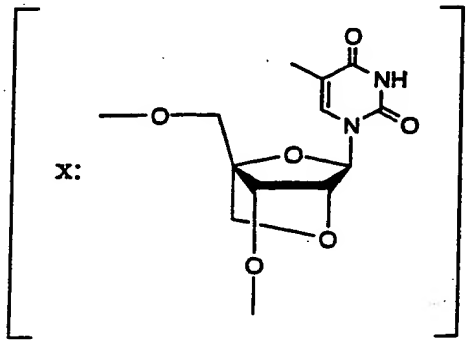
The four citations cited by the examiner contain a 2'-*O*,4'-*C*-methylene nucleoside having the following structure (I).



(I)

PZ2 was described in Example 142 in WO99/14226. PZ2, which contains the 2'-*O*,4'-*C*-methylene nucleoside of (I), was synthesized according to the method described in WO98/39352 and its nuclease resistance was compared with oligonucleotides containing the invented nucleosides. The purified PZ2 was analyzed by anion exchange HPLC (column: Tosoh TSKgel DEAE-2SW, 4.6 x 250 mm, solution A: 20% acetonitrile, solution B: 20%acetonitrile, 2M $HCOONH_4$, B% 20 – 60% (20 min), flow rate: 1 ml/min, column temp. 60°C, 260 nm). Retention time: 15.67 min. The structure of PZ2 was determined by negative-ion ESI mass spectroscopy. calcd: 4676.08, found: 4675.77.

PZ2: 5'-GC ATG TGC TGG AGA x-3'



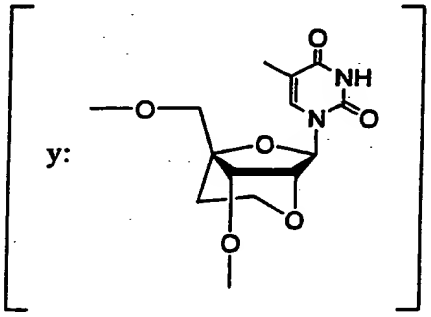
P2, which was described in Example 142 in WO99/14226, was synthesized according to the standard method. The purified P2 was analyzed by anion exchange HPLC (column: Tosoh TSKgel DEAE-2SW, 4.6 x 250 mm, solution A: 20% acetonitrile, solution B: 20%acetonitrile, 2M $HCOONH_4$, B% 20 – 60% (20 min), flow rate: 1 ml/min, column temp. 60°C, 260 nm). Retention time: 15.45 min. The structure of P2 was determined by negative-ion ESI mass spectroscopy. calcd: 4648.07, found: 4647.76.

P2: 5'-GC ATG TGC TGG AGA T-3'

(b) Synthesis of a compound of this invention

PZ2-ENA, which contains the invented nucleosides, was synthesized according to the method described in the specification of this application. The purified PZ2-ENA was analyzed by anion exchange HPLC (column: Tosoh TSKgel DEAE-2SW, 4.6 x 250 mm, solution A: 20% acetonitrile, solution B: 20%acetonitrile, 2M $HCOONH_4$, B% 20 – 60% (20 min), flow rate: 1 ml/min, column temp. 60°C, 260 nm). Retention time: 15.61 min. The structure of PZ2-ENA was determined by negative-ion ESI mass spectroscopy. calcd: 4690.11, found: 4689.80.

PZ2-ENA: 5'-GC ATG TGC TGG AGA y-3'



(c) Assay method of nuclease resistance of oligonucleotides and results

We tested according to the method described in Example 142 in WO99/14226. Briefly, Primers were 5' end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (Takara Bio, Japan). Reactions were boiled for 5 min after labeling to remove any PNK activity. 8 pmol of each primer was hybridized to 25 pmol of Template (sequence: 3'-ACG TAC ACG ACC TCT ACC TTG CTA-5') in 1x exonuclease buffer (Takara Bio, Japan , 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$ and 10 mM 2-mercaptoethanol). 10 units or 1 unit of Exonuclease III (Takara Bio, Japan) was added to each of the reaction. Controls were also set up which had 1 μl of water added in place of the enzymes. The reactions were incubated at 37°C for 5 min. The reactions were stopped by the addition of 10 μl of formamide/EDTA stop solution. The reactions were heated at 95°C for 3 min before loading onto a 19% polyacryamide 7M urea gel (1x TBE, 1400V, 1 hr). The gel was exposed

to an imaging plate for 15 min. The imaging plate was subjected to image analysis using a Bio Imaging Analyzer (BAS-2500, Fuji Photo Film Co., Ltd., Japan). The results are shown in Table 1.

Table 1. Percentage of remaining primers.

primers	10 units of exonuclease III	1 unit of exonuclease III
P2	4	6
PZ2	5	19
PZ2-ENA	9	60

(Discussion)

The image analysis showed that in the absence of the enzyme almost 100% of all primers (P2, PZ2 and PZ2-ENA) with full length remained. When the duplexes were incubated with 10 units of exonuclease III, which was the same condition as described in Example 142 in WO99/14226, we could not compare the cleavage rate because primers were cleaved very rapidly. When the amount of exonuclease III reduced to 1 unit, PZ2 of the prior art had a higher nuclease-resistance activity than P2, which is a natural oligothymidylate. Furthermore, although 19% of PZ2 was detected after 5 min of incubation, 60% of PZ2-ENA still remained. It turned out that RZ2-ENA of this invention had a much higher nuclease-resistance activity than RZ2 of the prior art. The remarkably high nuclease-resistance activity of a compound of this invention is not obvious for the person skilled in the art. Since the role of the ring structure of the invented nucleosides is not only structural, they are thought to have an inventive step in the light of the prior arts.

Certificate of Experimental Results (6)

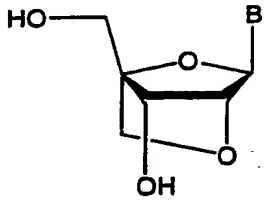
(Abstract)

Compounds of this invention were evaluated for stability in mouse and rat serum. From the results, it was clear that this invention has an inventive step in the light of the prior arts cited in the Official Report.

(Methods and Results)

(a) Synthesis of a reference compound

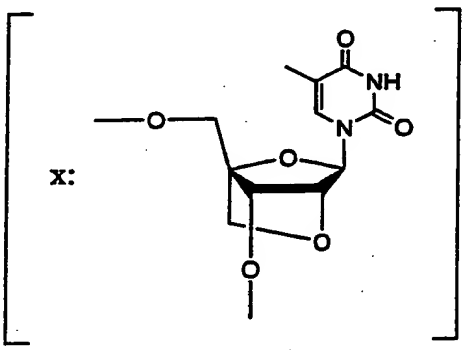
The four citations cited by the examiner contain a 2'-*O*,4'-*C*-methylene nucleoside having the following structure (I).



(I)

LNA 5'-$T^{L}_{13}T$ was described in D4, Singh *et al.* Chemical Communication, 4: 455-456, 1998. LNA 5'-$T^{L}_{13}T$, which contains the 2'-*O*,4'-*C*-methylene nucleoside of (I), was synthesized according to the method described in WO98/39352 and its stability was compared with an oligonucleotide containing the invented nucleosides. The purified LNA 5'-$T^{L}_{13}T$ was analyzed by anion exchange HPLC (column: Tosoh TSKgel DEAE-2SW, 4.6 x 250 mm, solution A: 20% acetonitrile, solution B: 20% acetonitrile, 2M $HCOONH_4$, B% 20 – 60% (20 min), flow rate: 1 ml/min, column temp. 60°C, 260 nm). Retention time: 16.92 min. The structure of LNA 5'-$T^{L}_{13}T$ was determined by negative-ion ESI mass spectroscopy. calcd: 4560.92, found: 4560.58.

LNA 5'-$T^{L}_{13}T$: 5'-xxx xxx xxx xxx xT-3'



A natural DNA T_{14} was synthesized according to the standard method. The purified DNA T_{14} was analyzed by anion exchange HPLC (column: Tosoh TSKgel DEAE-2SW, 4.6 x 250 mm,

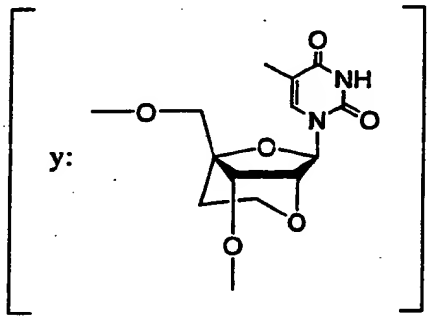
solution A: 20% acetonitrile, solution B: 20% acetonitrile, 2M $HCOONH_4$, B% 20 – 60% (20 min), flow rate: 1 ml/min, column temp. 60°C, 260 nm). Retention time: 14.66 min. The structure of T_{14} was determined by negative-ion ESI mass spectroscopy. calcd: 4196.78, found: 4196.66.

DNA T_{14}: 5'-TTT TTT TTT TTT TT-3'

(b) Synthesis of a compound of this invention

ENA 5'-T^{E}_{13}T, which contains the invented nucleosides, was synthesized according to the method described in the specification of this application. The purified ENA 5'-T^{E}_{13}T was analyzed by anion exchange HPLC (column: Tosoh TSKgel DEAE-2SW, 4.6 x 250 mm, solution A: 20% acetonitrile, solution B: 20% acetonitrile, 2M $HCOONH_4$, B% 20 – 60% (20 min), flow rate: 1 ml/min, column temp. 60°C, 260 nm). Retention time: 12.62 min. The structure of PZ2-ENA was determined by negative-ion ESI mass spectroscopy. calcd: 4743.27, found: 4742.77.

ENA 5'-T^{E}_{13}T: 5'-yyy yyy yyy yyy yT-3'



(c) Methods and Results Stability assay in mouse and rat serum

Mouse whole blood was obtained from adult male C57BL/6J mouse (Charles River Japan). Rat whole blood was obtained from adult male Sprague-Dawley rats (Charles River Japan). Put the blood at room temperature for 30 minutes and the serum separated by centrifugation at 3000 rpm for 10 min. A 10-μl aliquot of each oligonucleotide (1 mg / ml) was added to 490 μl of the mouse or rat plasma (finally 20 μg/ml) and incubated at 37°C. At the time points of 0, 0.5, 1, 2, 4, 6 and 24 hr, 50-μl aliquots were taken and immediately were added with ice-cold 100μl acetonitrile containing internal standard compound (5'-TTT TTT TTT TTT TTT TTT TTT TTT TX-3': X = 10-hydroxydecanylphosphate, T = thymidine, 5 μg/ml) and ice-cold distillated water. Then, samples were centrifuged at 15,000 rpm at 4°C for 3 min to remove the proteins. The supernatants were taken as samples for anion exchange HPLC analyses (column: Tosoh TSKgel DEAE-2SW, 4.6 x 250 mm, solution A: 20% acetonitrile, solution B: 20% acetonitrile, 2M $HCOONH_4$, B% 20 – 60% (20 min), flow rate: 1 ml/min, column temp. 60°C, 260 nm) The average residual amount of oligonucleotides were obtained from two independent experiments.

We compared the stability of LNA 5'-T^{L}_{13}T, DNA T_{14} and ENA 5'-T^{E}_{13}T in mouse and rat serum. Each oligonucleotide was incubated with mouse or rat serum (final conc. 98%) at 37°C and the mixture was analyzed by anion exchange HPLC. DNA T_{14} was completely degraded in 30 min.

While almost intact LNA 5'-$T^{L}_{13}T$ did not remain in mouse serum in 6 hr, more than 80% of intact ENA 5'-$T^{E}_{13}T$ remained as shown in Figure 1. While no intact LNA 5'-$T^{L}_{13}T$ remained in rat serum in 2 hr, almost all ENA 5'-$T^{E}_{13}T$ remained as shown in Figure 2. These results indicate that LNA 5'-$T^{L}_{13}T$ did not display stability in serum and that ENA 5'-$T^{E}_{13}T$ was more stable than corresponding LNA 5'-$T^{L}_{13}T$ in both mouse and rat serum.

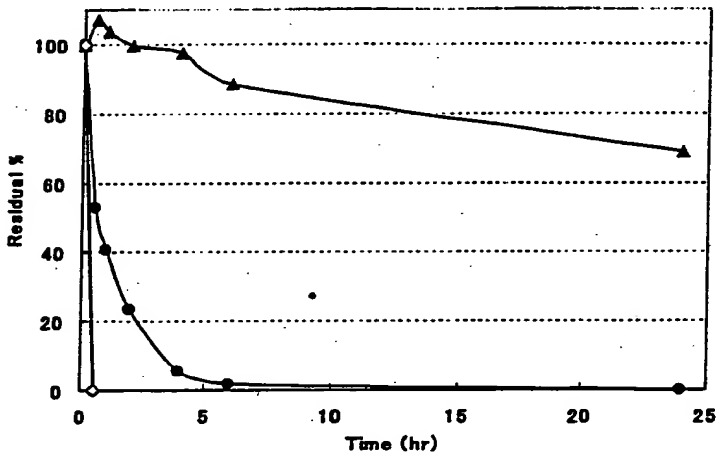


Figure 1. Stability of oligonucleotides in mouse serum.
Closed circles: LNA 5'-$T^{L}_{13}T$; open diamonds: DNA T_{14}; closed triangles: ENA 5'-$T^{E}_{13}T$.

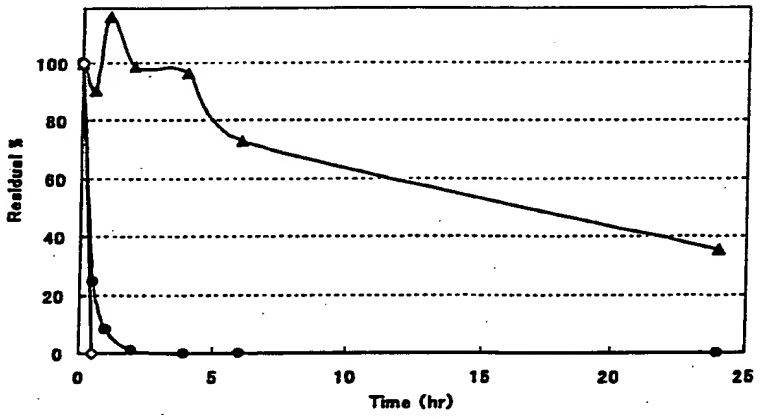


Figure 2. Stability of oligonucleotides in rat serum.
Closed circles: LNA 5'-$T^{L}_{13}T$; open diamonds: DNA T_{14}; closed triangles: ENA 5'-$T^{E}_{13}T$.